The Role of the Rbfox Proteins in Zebrafish Muscle Development

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ABSTRACT

Tissue specific alternative splicing is essential to the proper function of genes in many pathways. Muscle development is largely affected by alternative splicing of muscle-specific exons. Defects in muscle-specific splicing events in animal models lead to severe phenotypes reminiscent of muscular dystrophies in humans. Our lab has previously shown that depletion of two zebrafish genes, *rbfox1l* and *rbfox2*, results in changes in muscle-specific alternative splicing patterns of target genes, in muscular structural defects, and paralysis of the embryos. To further characterize the Rbfox-regulated alternative splicing, I strove to generate null mutations for both *rbfox* genes. I pursued targeted mutagenesis using zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPRs). Both ZFNs and TALENs were relatively ineffective but CRISPRs showed potential to induce mutagenesis. To prioritize potential Rbfox-regulated exons, I screened a list of putative target exons that the lab had identified by RNA-seq as mis-regulated in the double *rbfox1l*, *rbfox2* knockdown. Candidate exons were evaluated for the magnitude of the mis-regulation, the expression pattern of the gene, the function of the exon in the gene, and for Rbfox binding motifs in the adjacent intron. To date, my analysis has highlighted twelve exons, belonging to eleven genes, all of which showed both a significant depletion in the absence of the Rbfox proteins and a binding motif in the downstream intronic region.

INTRODUCTION

The Zebrafish Model System

The zebrafish (*Danio rerio*) is emerging as an important vertebrate model to study muscle development and disease (Gibbs et al., 2013, Goody et al., 2012, Seger et al., 2011). Zebrafish embryos are optically clear, can be grown in large numbers in a relatively small amount of space, are externally fertilized, and reach sexually maturity after only two months (Leischke and Currie, 2007, Santoriello and Zon, 2012). These advantages facilitate genetic manipulation and allow for mutants to be generated in a relatively short amount of time. Many muscular development studies have focused on muscular dystrophies and myopathies (Daya et al., 2014, Raeker et al., 2014, Vieira et al., 2014, Smith et al., 2014). Like mammals, zebrafish produce dystrophin, and knocking out dystrophin production results in muscle defects reminiscent of human muscular dystrophies (Bassett et al., 2003, Guyon et al., 2007). Furthermore, the transparency of zebrafish embryos has been used to follow and study muscle-specific gene expression in vivo (Myhre et al., 2014). For these reasons the zebrafish is an ideal model system in which to study genes that are important in muscle function and development.

Muscle Development in Zebrafish

Many important events in zebrafish muscle development occur within the first 30 hours post fertilization (hpf) and mutations in muscle-specific genes yield very noticeable phenotypes. Motoneurons are observed to grow extensively during this period and interact with muscle cells (Hanneman and Westerfield, 1996) while the slow muscle fibers undergo a massive migration from the notochord to lateral somite position in the embryo (Cortés et al., 2003, Devoto et al., 1996). This migration of the slow muscle is followed shortly by the elongation and

differentiation of fast muscles (Devoto et al., 1996). By 18-24 hpf, spontaneous muscle contractions of the embryo are evident and defects in the neuronal or muscular systems may be identified in embryos that do not exhibit the normal strength or timing of contractions (Granato et al., 1996, Grunwald et al., 1988). These observations allow for isolation of mutants with motility defects, identifying mutations in genes important for muscle or neuron development (Granato et al., 1996, Grunwald et al., 1988). To date, several zebrafish mutations have identified genes that when mutated in humans give rise to muscle disease, including autosomal recessive centronuclear myopathy (CNM2), limb-girdle muscular dystrophies (LGMD), and GNE Myopathy (Daya et al., 2014, Raeker et al., 2014, Vieira et al., 2014, Smith et al., 2014). These zebrafish models of human disease will allow researchers to study the disease in greater detail and to develop potential therapies.

Alternative Splicing Programs

Alternative splicing is a mechanism that produces multiple RNA transcripts, and thus proteins, from a single gene. Therefore, a limited number of genes can produce a diversity of protein products (Nilsen and Graveley, 2010). Some muscle defects can be linked to improper alternative splicing events of genes involved in regulating and promoting muscle formation and function (Bland et al., 2010, Gallagher et al., 2011, Hirata et al., 2007, Xu et al., 2002). There are four main types of alternative splicing: exon skipping (cassette exon), alternative 3' splice site, alternative 5' splice site, and intron retention (Keren et al., 2010). In cassette exon alternative splicing programs, entire exons are included or excluded in the produced RNA transcript. These alternative splicing programs depend upon the binding of the *trans*-acting splicing proteins to intronic or exonic target sequences of the pre-mRNA. The Rbfox proteins have been identified as such *trans*-acting splicing factors important in muscle development (Auweter et al., 2006, Jin et

al., 2003, Ponthier et al., 2006). Anomalies in muscle splicing programs have been linked to human muscular diseases including Duchenne Muscular Dystrophy (DMD) and spinal muscular atrophy (SMA) (Cooper et al., 2009).

Rbfox Proteins in the Alternative Splicing Program

In a cassette exon alternative splicing pathway, Rbfox proteins operate as splicing factors that facilitate the inclusion or exclusion of alternative exons (Auweter et al., 2006). Some of the Rbfox-regulated genes are known to be important to the development and function of muscle, including *fxr1* (Gallagher et al., 2011, Van't Padje et al., 2009). Rbfox proteins show a high and unique affinity for a 'UGCAUG' motif but also are found to bind to the pentamer 'GCAUG' (Auweter et al., 2006, Jin et al., 2003). This binding motif is prevalent in the downstream introns of exons in muscle-specific genes, suggesting that the Rbfox proteins could regulate the inclusion of these exons and therefore play a key role in muscle development and function (Bland et al., 2010, Das et al., 2007, Minovitzky et al., 2005, Sugnet et al., 2006, Zhang et al., 2008). In the cassette exon alternative splicing program, the Rbfox proteins bind in the downstream intronic regions to induce the inclusion of the upstream exon and in the upstream intronic regions to induce the exclusion of the downstream exon, a mechanism conserved between fish and humans (Lovci et al., 2013, Sun et al., 2012).

The Role of Rbfox1l and Rbfox2 in Zebrafish Muscle Development

Two of the *rbfox* genes, *rbfox1l* and *rbfox2*, are important in the splicing program of zebrafish heart and skeletal muscle genes (Gallagher et al., 2011). The *rbfox1l* gene is expressed exclusively in skeletal and cardiac muscle (Gallagher et al., 2011). The *rbfox2* gene is a paralog of *rbfox1l*, but is expressed more broadly (Gallagher et al., 2011). These genes are essential for proper splicing and muscle function in embryogenesis, since simultaneous antisense-mediated

knockdown of *rbfox1l* and *rbfox2* results in paralyzed embryos with severe developmental defects in skeletal and cardiac muscle and in broad changes in alternative splicing patterns (Gallagher et al., 2011). However, single knockdown of either gene results in either normal myofibril striation or very mildly altered striation, in contrast to the double knockdown, suggesting some redundancy of the program. Currently *fxr1*and *ube2d3*, among others, have been identified as muscle-specific genes that show significant changes in splicing patterns in the knockdown of the Rbfox1l and Rbfox2 proteins (Gallagher et al., 2011).

Targeted Genome Modification Methods

Although we have learned a lot about *rbfox* function from transient knockdown of the *rbfox* genes, a double null mutant is essential to fully characterize the role of the Rbfox1l and Rbfox2 proteins, especially in later development and beyond into adulthood. For my thesis, I pursued several established reverse genetics approaches to create a null mutation in the *rbfox1l* and *rbfox2* genes. First, both zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) were used to target the *rbfox* genes. ZFNs and TALENs rely on nucleases engineered to target a specific sequence and then cleave the double-stranded DNA (*Figure 1*, Amacher, 2008, Huang et al., 2012). ZFN modules bind to three base pairs while TALEN modules bind to only one base pair (*Figure 1B*). The induced double-stranded break can then be repaired by either the homology directed repair (HDR) or the non-homologous end joining (NHEJ) pathway (*Figure 1A*). A donor DNA can be used as a template to induce mutagenesis through the HDR pathway. NHEJ tends to be naturally error-prone and often results in small insertions or deletions, and thus in a frame-shift mutation. While both of these strategies rely on recognition of the DNA by a nuclease protein, ZFNs tend to have a higher cost and a lower efficacy in inducing mutagenesis in the germ-line than TALENs (Blackburn et al., 2013).

The CRISPR/Cas9 system is a new approach to targeted mutagenesis in zebrafish that is much easier and cheaper to generate than ZFNs or TALENs (Li et al., 2013). This system operates through the recruitment of the Cas9 endonuclease by a guide RNA to a target DNA sequence (*Figure 2*). The recruited Cas9 then cleaves the double-stranded DNA in the targeted sequence, which can be rejoined by error-prone NHEJ (Waaijers and Boxem, 2014). This approach has been shown to provide reliable knock-out and is emerging as a viable method of introducing knock-in (Hruscha et al., 2013, Schmidd and Haass, 2013). Given the reliability demonstrated and the cost-efficiency, CRISPRs are quickly becoming a preferred method for generating stable mutants in zebrafish.

Putative Target Exons of Rbfox Identified by RNA Sequencing

In order to further study how Rbfox-mediated alternative splicing directly impacts the development and function of muscle in zebrafish, characterizations of the exact targets are needed. Our lab generated RNA sequencing data from the double knockdown zebrafish that identified putative Rbfox-regulated target exons (Thomas Gallagher and Sharon Amacher, unpublished). In collaboration with another lab, the RNA-seq data was analyzed using the DEXseq method (Anders et al., 2014, Thomas Gallagher and John Conboy, unpublished). I screened the downstream introns of these exons for the Rbfox binding motif. Screening for putative binding motifs will narrow the search for Rbfox target exons and facilitate further connections to be made between Rbfox proteins and their target genes. The short list of high priority exons I generated will make analysis of the null mutants more focused. Prior to analysis with the mutants, the genes identified will allow for general trends of localization and function of putative Rbfox target genes to be revealed.

Goal of My Thesis Project

The long-term goal driving my thesis project is to understand the role of alternative splicing in vertebrate development. In my thesis, I used Rbfox proteins in zebrafish muscle development as a model to study alternative splicing in a vertebrate system. I strove to generate a null mutation in two known splicing factors, *rbfox1l* and *rbfox2,* and to identify putative Rbfox-regulated exons in order to take an important step towards my long-term goal. One of my motivations for pursuing a null mutant was the ability to study the effects of an Rbfox protein knock out in muscle development of later embryos and adults. Although double knockdown of *rbfox1l* and *rbfox2* via morpholino injection yields a paralyzed phenotype with great changes in target gene splicing patterns, to completely characterize the *rbfox* phenotype a null mutant is necessary and was therefore a huge goal of my project. In addition to a genetics approach, molecular studies are needed to show the direct connection between Rbfox-regulated exons and muscle development. As a step toward these studies, I screened through a list of putative target exons generated from other members of the lab using an RNA sequencing study comparing the transcriptomes of single and double *rbfox* knockdown. My work created a short list of high priority putative target genes for future study. Although not all the goals of my project were met, I learned a great deal about reverse genetics approaches, vertebrate muscle development, and the role of alternative splicing programs.

MATERIALS AND METHODS

Fish Husbandry

Live wild-type (AB) and putative mutant adult fish were kept at 28.5°C on a 14 hour light/10 hour dark cycle. Embryos were collected from natural spawnings between live adult fish and then raised at temperatures between 22°C and 30°C.

TALEN mRNA Injection Stock Preparation and Microinjection into Zebrafish Embryos

DNA encoding *rbfox1l*-targeting TALENs was cloned into bacteria via a plasmid vector. Bacteria were grown at standard conditions and plasmid DNA was extracted using the Qiagen Miniprep Kit. 4 ug of purified plasmid was linearized with the NotI restriction enzyme (NEB) and purified using the MinElute Enzyme Reaction Cleanup Kit (Qiagen). 1 ug of linearized template was used for the transcription of TALEN encoding-m RNA with the Ambion® mMessage mMachine T7 kit. RNA was prepared in concentrations of 50 pg/nl, 100 pg/nl, 150 pg/nl, and 200 pg/nl for injections. This RNA was injected into one-cell stage embryos.

Between 3 and 7 nl of a given TALEN RNA stock was micro-injected into one-cell stage embryos (within 20 minutes post fertilization). Embryo clutches were collected roughly every 20 minutes. Embryos were then kept between 25°C and 38.5°C for further analysis.

CRISPR mRNA Injection Stock Preparation and Microinjection into Zebrafish Embryos

The CRISPR injection stock was comprised of the guide RNA that contains base complementarity to the target locus and the Cas9 mRNA. I first identified guide RNA target sequences for *rbfox1l* and *rbfox2* using the CRISPR Design Tool (www.genome-engineering.org/crispr) (Hsu et al, 2013). Complementary DNA oligonucleotides encoding the

guide RNA were designed that contained compatible overhangs for cloning into the guide expression vector pDR274 (Addgene). Cloning was performed by linearizing pDR274 with BsaI-HF, ligation with the complementary guide DNA oligonucleotides with T4 DNA ligase (NEB) and transformation into DH5a competent cells (Life Technologies). Plasmids were sequence confirmed and then used to synthesize RNA using the Life Technologies™ Maxiscript T7 kit. Transcribed RNA was precipitated by lithium chloride (1/10 volume 7.5 M LiCl + 2 volumes 100% ethanol) and washed with ethanol (1 ml, 70%) and stored at -80°C for 48 hours before spinning DNA down and resuspending in 10 µl of Hypure H_2O (Thermo Scientific) or Buffer EB (Qiagen).

To generate the Cas9 mRNA, a frozen pCS2-nCas9n was acquired from AddGene and cultured in order to prepare a midiprep for linearization using NotI-HF (NEB). After confirming the clones by sequencing, the *cas9*-encoding plasmid was transcribed using the Ambion® mMessage mMachine Sp6 kit from Life Technologies™. The mRNA was purified using lithium chloride precipitation and stored at -80°C.

A single nucleotide polymorphism (SNP) was found in the sequence targeted by this *rbfox1l* guide RNA (*see Figure 3*). This SNP can be either a 'C' or a 'T' in wild-type fish. This guide RNA targeted a sequence containing a 'C'. Both the 'C' and the 'T' allele were found to be present in the stocks of the fish used in this project.

Initial injection stocks (10 µl) of *rbfox1l*-CRISPR contained a guide RNA concentration of 33 ng/µl and a Cas9 mRNA concentration of 6 ng/µl. Secondary stocks of *rbfox1l* contained a guide RNA concentration of 33 ng/µl and a Cas9 mRNA concentration of 66 ng/µl. Final *rbfox1l*

stocks and initial stocks of *rbfox2* contained a guide RNA concentration of 70 ng/µl and a Cas9 concentration of 66 ng/µl. Injection stocks were stored at -80°C.

Between 3 and 7 nl of a given CRISPR stock was injected into one-cell stage embryos (about 20 minutes post fertilization) under a light microscope. Embryo clutches were collected roughly every 20 minutes. Embryos were then kept between 25°C and 28.5°C for further analysis.

Preparation of DNA from Embryos and Fin Clips

For fin preparations, adult fish were anesthetized using a solution of Tricaine methanesulfonate (0.004%), which was prepared by adding 4.2 mL of Tricaine methanesulfonate (4mg/mL) in 100 mL of H_20 from the aquatic system. The last ⅓ of the tail fin was clipped using dissecting scissors disinfected in ethanol (70%). The fin clip was then transferred to a tube of 50 µl of 1X ThermoPol® Buffer (NEB) on ice. Fish were recovered in 3 L of system water before being returned to the system. For embryo preparations, single 24-72 hpf embryos were dechorionated under a light microscope using fine forceps. Using a fire-polished Pasteur pipet, embryos were transferred to a tube of 50 µl of 1X ThermoPol® Buffer (NEB).

Embryos or fin clips in 1X ThermoPol® Buffer (NEB) were heated to 95°C for 10 minutes and then cooled to at least 55°C in a thermocycler. Proteinase K (5 µl, 10 µg/µl) was added to each sample. The samples were then incubated at 55°C for 1-3 hours and heated to 95°C to denature the Proteinase K. Samples were then cooled to at least 4°C and spun on a tabletop centrifuge for 1 minute to isolate DNA in the supernatant and pull down any lasting debris. DNA was then taken from the supernatant for further analysis. Remaining DNA was stored at -20°C.

High Resolution Melting Analysis (HRMA)

The procedure for using HRMA to screen zebrafish DNA was adapted from the procedure outlined by the Grunwald lab (Dahlem et al., 2012). DNA was amplified using primers specific to a given exon sequence (*Table 1*). DNA was prepped and analyzed using EvaGreen® (BioRad Laboratories, Inc.) fluorescence based amplification in a Bio-Rad CFX Real-Time Machine according to the recommendations provided from Bio-Rad. The amplification of the DNA was achieved using Precision Melt Supermix (BioRad) diluted to a final concentration of 1X with 1 ul of genomic DNA and 0.5 ul of 10 um forward and reverse primers designed to generate an amplicon no greater than 150 base pairs (bp). Cycling conditions were as follows: denaturing temperature of 95°C (15 s), annealing temperature of 60°C (15 s), and extension temperature of 72°C (15S), cycled for a total of 45 times.

Primer	Forward Primer Sequence	Reverse Primer Sequence	Target Sequence
ZFN Primers			
rbfox1l-HRM	5'_AGCGCTGCTCATCCAAAT_3'	5'_caggtgatcagtcttacCGTGT_3'	5'_GGCCCTCAGCATGACGGGACG GAGTCT_3'
TALEN Primers			
rbfox1l-TAL	5'- TCCCGTCCCTTTTAGCTGTT -3'	5'- TTTACCTGAACAATGCCGGG -3'	5'_TGTCTTCTCCTACTGTGATCCTT CAGCCCTACGGACTCCCAGTTTAC CCTCA_3'
rbfox2-TAL	5'- CATGTGTGGTGAGGAGGGC -3'	5'- TTCTGTGGAGGAGGAGGAG -3'	5'_GCAGTGGTCCCGTTCTTCCCCC CTCCT_3'
CRISPR Primers			
rbfox1l-HRM-3	5'- GCGCTGCTCATCCAAATTCA -3'	5'- CGTGTTACTGGCAGTTATAGAC -3'	5'_GGGCCCTCAGCACGACGGGA_3 '
rbfox2-HRM-2	5'-TCCTCCTCCTCCACAGAATG -3'	5'- TGCTGGGGTTGGAGGCTC -3'	5'_GGGCAGACGGAGGCGGTGGC_ 3'

Table 1. Primers used in both the TALENs and CRISPRs approach to inducing mutagenesis. Note that the highlighted nucleotide in the *rbfox1l*-CRISPR target sequence represents a single nucleotide polymorphism in wild-type zebrafish that can be a 'C' or 'T'.

Sequencing DNA

DNA was prepped with an M13 Forward primer according to the Ohio State Plants-Microbe Genomics Facility (PMGF) guidelines and submitted to the PMGF for sequencing.

Bioinformatics

Both *rbfox1l* and *rbfox2* were knocked down by morpholinos by Thomas Gallagher at doses of 6 ng for each morpholino. Total RNA was extracted at 26 hpf using Trizol according to the manufacturer's instructions (Life Technologies™) and mRNA was further purified using oligo-dT magnetic beads (Life Technologies). mRNA was fragmented using Ambion's chemical fragmentation kit and reverse transcribed with random primers according to the manufacturer's instructions for the reverse transcriptase SuperScript III (Life Technologies). cDNA libraries were generated using the Apollo 324 robotic preparation system (Wafergen) 100 bp paired end reads were then performed on Illumina's HiSeq 500, generating greater than 200 million reads per library. In collaboration with Susan Celniker and Ben Brown (Lawrence Berkeley National Laboratory), the raw sequencing data was assembled and mapped to the zebrafish Zv9 genome assembly. Following mapping and assembly, the transcriptomes of single and double knockdowns were analyzed using the DEXseq method (Anders et al., 2014) to identify up- and down-regulated exons. The results of this analysis were analyzed in the zebrafish (*Danio rerio*) Zv9 UCSC genome browser, http://genome.ucsc.edu/cgi-bin/hgGateway. Downstream intronic regions of these exons were screened for the 'UGCAUG' motif. DNA sequences were taken from UCSC Zv9 genome browser. Information from identified genes of interest was taken from the ZFIN website, http://zfin.org/.

RESULTS

Ineffective Mutagenesis of the *rbfox1l* gene through the use of Zinc-Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs)

To pursue *rbfox1l* null mutations, other lab members had injected ZFNs and generated putative founders, F1, and F2 progeny. Founders (n=7), F1 (n=40), and F2 (n=25) progeny were

screened by HRMA and were found to consistently cluster with wild-type curves (*data not shown*). The deflection that had previously suggested that these fish were mosaic founders was found to represent a SNP in the amplified region.

The *rbfox1l* TALENs (TAL3155/TAL3154) were obtained from the plasmid repository Addgene via a large-scale TALEN generation effort by the laboratory of Keith Joung (Massachusetts General Hospital; Sander et al, 2011). These TALENs were designed with DNA binding specificity against exon 2 of the *rbfox1l* locus (*Figure 4*). Since we suspected that *rbfox1l* has alternative start sites, like its orthologs in mouse and human, and the RNA recognition motif (RRM) is found in exon 3, exon 2 appeared to be the best target for mutagenesis. By targeting exon 2, a frame shift mutation is expected to disrupt the remainder of exon 2 and all of the RRM in exon 3 and therefore result in a non-functional protein. The TALEN-encoding plasmids were linearized by PmeI restriction digestion (*Figure 4A*) and used to transcribe the TALEN-encoding mRNAs, which was confirmed by a diagnostic gel (*Figure 4B*). Four different injection stocks of *rbfox1l* TALENs (250 pg, 500 pg, 750 pg, and 1,000 pg) were made and injected into over 450 embryos (n = 20, 83, 270, and 95, respectively). None of the embryos injected with any concentration showed a significant deflection from the wild-type uninjected embryos when analyzed by HRMA, indicating that these TALENs or my injection technique were ineffective at causing mutagenesis at the *rbfox1l* locus (*Figure 5*).

Mutagenesis of the *rbfox1l* gene using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 System

In order to take a step in generating a null mutant for characterization of the Rbfox-regulated splicing program, the *rbfox1l* gene was targeted for mutagenesis through the use of the CRISPR-Cas9 system. A guide RNA targeting exon 2 of the *rbfox1l* gene was designed using the

CRISPR Design Tool (Hsu et al, 2013) and generated by synthesis of complementary DNA oligonucleotides with sequence specificity for the target locus. Successful cloning of the guide-encoding plasmids was confirmed by both sequence and gel analysis (*Figure 6*). The diagnostic gel showed a vector band and tight insert band at the expected sizes of 1,861 base pairs and 289 base pairs, respectively (*Figure 6C*). After construction of the *rbfox1l*-CRISPR, we discovered that the targeted region of exon 2 contains a single nucleotide polymorphism (SNP) of either a 'T' or 'C'. Since the SNP is within the target sequence, it may interfere with the effectiveness of the guide RNA. Our *rbfox1l*-CRISPR targeted the sequence containing a 'C' (*Figure 6A*). Since the CRISPR design tool from CRISPR Genome Engineering Resources designated this site as one of the top sites to target and the CRISPR was already made, we proceeded to inject this guide RNA.

To address the issue of the SNP, stocks of wild-type (AB) fish were pre-screened using high resolution melt analysis (HRMA) to produce groups of fish that contained the same SNP genotype. The *rbfox1l* guide RNA (165 pg), with Cas9 RNA (30 pg), was injected into the offspring of pre-screened wild-type zebrafish that lacked the SNP previously mentioned. I then screened these embryos by HRMA for evidence of somatic mutagenesis at the targeted site (*Figure 7*). Only one out of these nineteen injected embryos showed mild deflection from the wild-type uninjected controls (*Figure 7B*). HRMA melt curves can be temperature shifted to superimpose the curves at a certain fluorescence intensity to help distinguish between curves. The mild deflection was maintained in the temperature shifted analysis, suggesting that the difference is real, but was too mild to suggest mutagenesis (*Figure 7*).

These experimental results could suggest that my injection technique was insufficient or that a component of the injection stock was defective. Other anecdotal evidence from lab

members suggested that the Cas9 mRNA was too dilute to work properly. To address this issue I acquired a guide RNA targeting the *pnrc2* gene known to be effective at inducing strong deflections in HRM analysis. Using this confirmed *pnrc2* guide RNA (165 pg) and more concentrated Cas9 mRNA (330 pg), I generated a new injection cocktail. This was injected into the offspring of wild-type fish and then analyzed by HRMA (*Figure 8*). These embryos showed distinct deflection consistent with previous *pnrc2* HRM analysis of CRISPR injections (*Figure 8A*). This result demonstrated that both my injection technique and this dose of Cas9 mRNA were sufficient to induce strong deflection in HRM analysis.

This confirmed Cas9 was then used to generate an injection stock of guide *rbfox1l* RNA (165 pg) and more concentrated Cas9 mRNA (330 pg) that was injected into embryos obtained from group matings of wild-type parents that had not been pre-screened for the target site SNP. The embryos thus represented all three possible SNP genotypes and could therefore be used to analyze the effectiveness of the CRISPR-Cas9 system on each genotype. The HRMA showed mild, yet consistent, deflection in injected embryos from one of the three SNP genotypes (*Figure 9A*). Since this guide RNA matched the 'C' SNP, it was expected that this *rbfox1l*-CRISPR would have a greater efficiency at recognizing the DNA of fish containing the 'C' SNP. These HRMA results may reflect the greater efficiency of the *rbfox1l*-CRISPR at targeting the homozygous 'C/C' SNP versus the heterozygous 'C/T' or homozygous 'T/T' SNP.

To further test the ability of the CRISPR-Cas9 system, an injection stock was prepared of higher guide RNA concentration (350 pg) and the same Cas9 concentration (330 pg) as this Cas9 concentration was shown to be sufficient in producing *pnrc2* mutagenesis. I injected this cocktail into the offspring of Tg(*cmlc2-gCaMP6.1*) adult fish because this stock yields great numbers of fertile embryos. The Tg(*cmlc2-gCaMP6.1*) line are wild-type at the *rbfox1l* locus and were not

pre-screened for the SNP. Analysis of these injected embryos showed that the *rbfox1l*-CRISPR is probably effective at inducing mosaic somatic mutagenesis in zebrafish embryos (*Figure 10*). Ten embryos, belonging to two of the SNP genotypes, out of the fifteen embryos screened showed altered patterns in the HRMA curves in addition to mild deflection compared to uninjected embryos, suggesting that these embryos hold somatic mosaic mutations. This result indicates a somatic mutagenesis rate in injected embryos of 66.7%.

In the SNP genotype seen in Figure 10A, four out of the eight injected embryos screened showed distinct differences in the melting patterns compared to uninjected embryos. These differences were maintained in the temperature shifted analysis, indicating a somatic mutagenesis rate in the injected embryos of 50%. These results suggested that there are changes induced by the injections in the target sequence of the *rbfox1l* gene. Similarly, differences were seen in the patterns of all six of the embryos seen in the SNP genotype of Figure 10B, suggesting a somatic mutagenesis rate in injected embryos of 100%. This further supported that there is potential mutagenesis in these embryos. It is likely that the SNP genotype of embryos analyzed in Figure 10B represents the homozygous SNP genotype 'C/C', which this *rbfox1l*-CRISPR is designed to target. Following this logic, it is likely that the SNP genotype corresponding to the injected embryos of Figure 10A is the heterozygous 'C/T' SNP genotype that would be less efficiently recognized by this *rbfox1l*-CRISPR system.

Mutagenesis of the *rbfox2* gene using the CRISPR-Cas9 System

The *rbfox2* gene was also targeted for mutagenesis through the use of the CRISPR-Cas9 system. A guide RNA targeting exon 2 of the *rbfox2* gene was designed using the CRISPR Design Tool (Hsu et al, 2013) and generated by synthesis of complementary DNA oligonucleotides with sequence specificity for the target locus. This guide RNA plasmid was

then confirmed by sequencing (*Figure 11B*). The guide RNA (350 pg) and Cas9 mRNA (330 pg) were co-injected into 50 embryos collected from crosses between Tg(*cmlc2-gCaMP6.1*) fish. The Tg(*cmlc2-gCaMP6.1*) fish were used for purposes as described above. A subset of these injected embryos and uninjected control embryos were analyzed by HRMA. Two of ten embryos analyzed carry potential mutagenesis (*Figure 12*). The DNA of the injected embryos had melt curves that deflected from wild-type by approximately 0.15 RFU when normalizing the melt curves against wild-type samples. The deflections were held in the temperature shifted analysis (*Figure 12B*). If these embryos are confirmed by sequencing to have mutations, this would correspond to a somatic mutagenesis rate in injected embryos of 66.7%. Unfortunately, DNA from only three of the injected embryos amplified well enough to be analyzed, leaving the possibility that a different rate of mutagenesis may actually be induced.

Identification of Putative Alternative Exons Regulated by the Rbfox Proteins

RNA sequencing data was generated from the double knockdown of *rbfox1l* and *rbfox2* by our lab using morpholinos (Thomas Gallagher and Sharon Amacher, unpublished data). In collaboration with the Celniker lab, the transcriptomes of the single and double knockdown fish were analyzed using the DEXseq method to identify exons that were either up regulated or down regulated in the knockdown of the Rbfox1l and Rbfox2 proteins (Anders et al., 2014). This software identified 596 down-regulated and 403 up-regulated exons from this transcriptome-level analysis. The top twenty down-regulated exons were screened for the 'UGCAUG' binding motif in the downstream intron. Chromosome coordinates were cross-referenced with the UCSC genomic database to obtain the intron DNA sequences.

Twelve exons, belonging to eleven genes, were found to have at least one putative Rbfox binding motif in the downstream intron (*Table 2*). Of the remaining eight exons screened, five

contain the pentameric ‘GCAUG’ sequence. The pentamer sequence indicates that Rbfox proteins may still bind directly to these targets based on evidence of zebrafish Rbfox1l binding to this pentamer in in vitro SELEX experiments (Jin et al., 2003). Interestingly, all ten of the ‘GCAUG’ sequences found in these five genes were preceded by an ‘A’ to give the sequence ‘AGCAUG’, which recent work using Surface Plasmon Resonance (GE Healthcare) suggests is the preferred Rbfox-binding motif after ‘UGCAUG’ (Thomas Gallagher and John Conboy, unpublished). The remaining three exons screened either did not have the hexameric or pentameric binding motifs or were the last exons of the gene and would require analysis of the 3’ UTR, which was not the focus of this analysis.

The $\log_2$fold value for each of the top twelve exons was less than -1.0, indicating a fold change of at least 2. The greatest fold change seen was that of *sarcalumenin*, which was found to be down-regulated 10.5 fold in the double *rbfox* knock down. I then looked these genes up in the ZFIN database to determine expression patterns and functions (*Table 2*). Many of these genes are known to be expressed in myotomes or neural tissue and some have known functions in muscle or motoneuron development.

Gene	Full Name	Targeted Exon	Log_2fold	Fold change	Expression	Function
srl	*sarcalumenin*	4	-3.40	10.57	heart, myotomes, somites	GcPase activity
eef1da	*elongation factor-1 delta a*	2 (ENSDART00000131905)	-3.28	9.70	not spatially restricted, myotomes	translation elongation factor activity
ube2d3	*ubiquitin-conjugating enzyme E2D 3*	6	-3.04	8.23	myotomes, neural tissue	ligation activity
eef1db	*elongation factor-1 delta b*	4	-2.80	6.97	not spatially restricted, myotomes	translation elongation factor activity
eef1da	*elongation factor-1 delta a*	3 (ENSDART00000002962)	-2.54	5.81	not spatially restricted, myotomes	translation elongation factor activity
fxr1	*fragile X mental retardation*	15 (ENSDART00000149482)	-1.91	3.77	myotomes, muscle, neural tissue	heart and muscle development,
scrib	*scribbled homolog*	27 (ENSDART00000021013)	-1.74	3.34	not spatially restricted, myotomes	protein binding, motor neuron
naca	*nascent polypeptide-associated complex alpha polypeptide*	2	-1.67	3.19	cardiac and skeletal muscle, whole organism	protein transport, myofibril
limch1a	*LIM and calponin homology domains 1a*	11 (ENSDART00000113023)	-1.65	3.15	trunk vasculature	actin binding, actomyosin structure
ctdsplb	*CTD (carboxy-terminal domain, RNA polymerase II,*	3 (ENSDART00000146600)	-1.61	3.06		phosphatase activity
ptk2.1	*protein tyrosine kinase 2 beta, b*	2 (ENSDART00000134302)	-1.29	2.45		protein kinase activity
cd99l2	*CD99 antigen-like 2*	6 (ENSDART00000079245)	-1.13	2.19	neural tissue, central nervous system	cell adhesion

Table 2. Table of putative target genes of the Rbfox proteins. Information for each gene was taken from the ZFIN database. Some of the identified exons were only present in the Ensembl prediction. In these cases the transcript ID from Ensembl has been included.

DISCUSSION

Mutagenesis of the *rbfox1l* and *rbfox2* genes using the CRISPR-Cas9 System

Current results from injections of the CRISPR-Cas9 system suggest that this method will successfully induce mutagenesis in the zebrafish *rbfox1l* and *rbfox2* genes. This system was shown to be effective at inducing mild, yet consistent, deflection at *rbfox* loci. The injected embryos currently growing up need to be screened by HRMA to identify putative mutants once they are at least 2 months old and then sequenced to confirm mutagenesis. In addition, higher doses of the *rbfox* CRISPRs should be injected into greater numbers of embryos in order to establish a dose curve by HRMA analysis and provide more embryos to screen for putative mutants. A CRISPR-Cas9 system targeting the *rbfox1l* target sequence containing the 'T' SNP has been acquired and should be prepped for injection. An additional *rbfox1l* CRISPR targeting a different, SNP-free, sequence of exon 2 could be designed and used to generate a null mutation.

Once identified and confirmed, fish with somatic mosaic mutations should be crossed to wild-type fish in order to segregate any germ-line mutations in individual offspring. These offspring should then be screened by HRMA and sequenced to screen for embryos that have a null mutation passed on from the founder parent. These F1 fish should be crossed again to wild type fish to perpetuate the null mutations. The F1 fish may also be crossed to each other to generate trans-heterozygous fish, which can then also be analyzed phenotypically. The embryos should be screened and separated into heterozygous F2 populations that could be used to obtain homozygous F3 fish to be analyzed phenotypically. When these F3 fish are successfully generated for both *rbfox* genes, doubly heterozygous fish will be crossed together to generate double mutant embryos for analysis.

Many phenotypic attributes of these fish should be analyzed, including heart size and muscle development. As the double knockdown of *rbfox1l* and *rbfox2* resulted in paralysis and an engorged heart in some embryos, these tissues should be analyzed in single and double knock out embryos. One way to analyze these tissues would be by live imaging using a transgenic reporter, such as with the myocardial reporter line *Tg(myl7-EGFP)*, or by immunohistochemistry in fixed embryos as demonstrated in the transient knockdown (Gallagher et al., 2011). The most severe defects would be expected in the double mutant skeletal and heart muscle. The transcriptomes of both the single and double mutants could be analyzed to identify up-regulated and down-regulated muscle-specific isoforms whose expression is dependent on Rbfox proteins. In addition to the RNA sequencing data already analyzed, further data could be collected in true knock outs, which would be expected to yield more dramatic fold changes.

If no mutations are found in the fish already injected, a new CRISPR targeting a different site on exon 2 or a different exon may be more effective. This new CRISPR may still be generated by the same algorithms previously used, but the target sequence should be selected in a way to prevent known SNPs and similarity with other chromosomes.

Ineffective Mutagenesis of the *rbfox1l* gene using ZFNs and TALENs

The embryos injected with either ZFNs or TALENs did not show significant deflections from wild-type embryos when analyzed by HRMA, suggesting that injection of the designed ZFN or TALEN pair is ineffective for targeted mutagenesis at the *rbfox1l* locus. The greatest deflection seen was around 0.10 RFU when normalizing fluorescence against wild-type control samples, which has been shown previously to not correlate with mutations when sequenced (unpublished data, Jared Talbot of Amacher lab). Preliminary analyses in our lab indicated that a value of at least 0.15 RFU is considered to be significant and indicative of mutagenesis that can

be recovered in the germ line. The ineffectiveness of these TALENs is not likely due to degradation of the injection stock as the RNA was shown on a gel to run at the expected size in tight, bright bands and to yield relatively high concentrations on the spectrometer. However, since CRISPR has been more effective in our lab's experience and CRISPR are relatively easy and cheap to generate, ZFNs and TALENs are no longer a practical approach to use to generate an *rbfox* null mutation.

Identification of Putative Target Alternatively Spliced Exons of the Rbfox Proteins

The putative Rbfox-regulated target exons already identified have much potential for further analysis. Additionally, the remaining exons generated by the transcriptome analysis should be analyzed. As for the eleven identified genes, the function of each of the Rbfox-regulated transcript isoforms from these genes has the potential to be important in muscle or neural development. We were not surprised that *fxr1* was at the top of this list as it is depleted in the double *rbfox* knockdown and these results are duplicated when the putative Rbfox binding motifs in the downstream intron of the regulated *fxr1* exon is blocked by target protecting morpholinos (Gallagher et al., 2011) Additionally, *fxr1* is essential to proper heart function and muscle formation in zebrafish. Depletion of *fxr1* by morpholinos results in cardiomyopathy and muscular dystrophy (Van't Padje et al., 2009). Similarly, we were excited to find the muscle-specific *naca* exon on this list since knockdown of *skeletal naca* (*sknac*), the muscle-specific alternatively spliced *naca* isoform, results in muscular defects and paralyzed embryos in zebrafish (Li et al., 2009).

The analysis of putative Rbfox target exons may be approached by many different methods. These exons could be analyzed in the *rbfox1l*, *rbfox2* double mutants by in situ

hybridization and Western blots to detect any down regulation that would support that they are impacted by the Rbfox proteins in a way that can be visualized. The identification of the Rbfox binding motifs in both *fxr1* and *naca* is essential to generate RNA probes for future use in an RNA Electrophoretic Mobility Shift Assay (RNA-EMSA) or an RNA pull-down assay. Both of these techniques could be used to show direct binding of the Rbfox proteins to the introns in vitro to further support that the Rbfox proteins regulate muscle development through the alternative splicing of target muscle-specific genes. In addition to these experiments, *fxr1*, *naca*, and any other muscle-specific exons that are identified should be tested for its ability to rescue the double *rbfox* knockdown embryos. Even partial rescue would further support the role of the Rbfox proteins in contributing to proper muscle development through a cassette exon alternative splicing program.

Although not every goal of this thesis project was met, the CRISPR work contributed to the generation of *rbfox* null alleles and the results of the putative target exon screen provided evidence supporting that the Rbfox-regulated alternative splicing is important in muscle development.

FIGURES

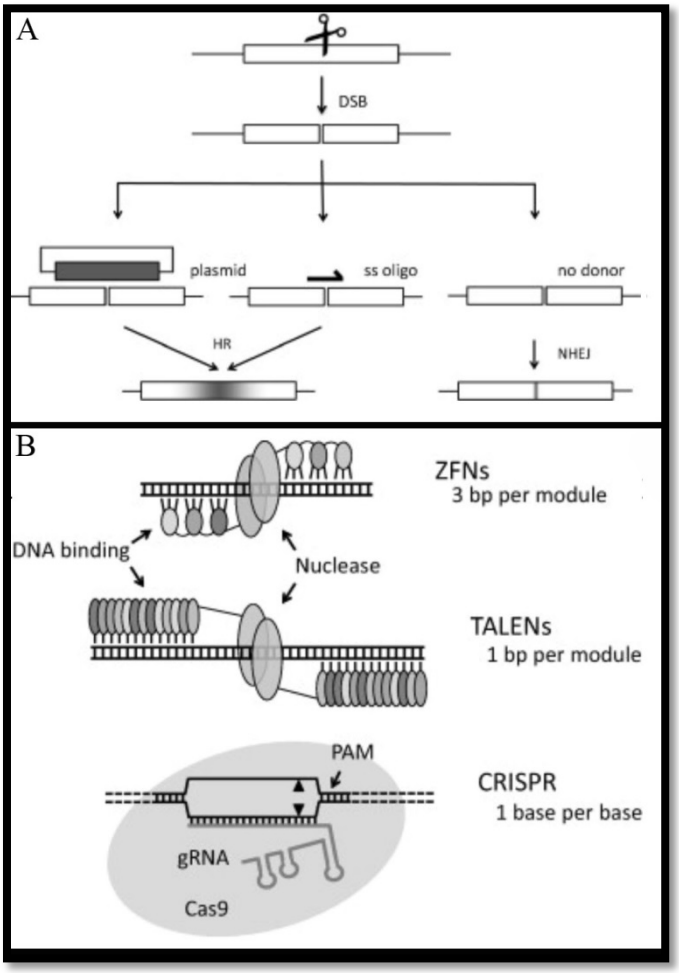

Figure 1. (From Methods of Beumer and Carroll, 2014) (A) Double-stranded breaks can be repaired by either non-homologous end joining (NHEJ) or homologous recombination (homology directed recombination, HDR). Mutagenesis by HDR requires donor DNA to act as a template while errors by NHEJ occur naturally. (B) All three approaches to mutagenesis, ZFNs, TALENs, and CRISPRs, rely on inducing double-stranded breaks. The specificity of both ZFNs and TALENs involve protein-DNA recognition while CRISPRs involve RNA-DNA recognition.

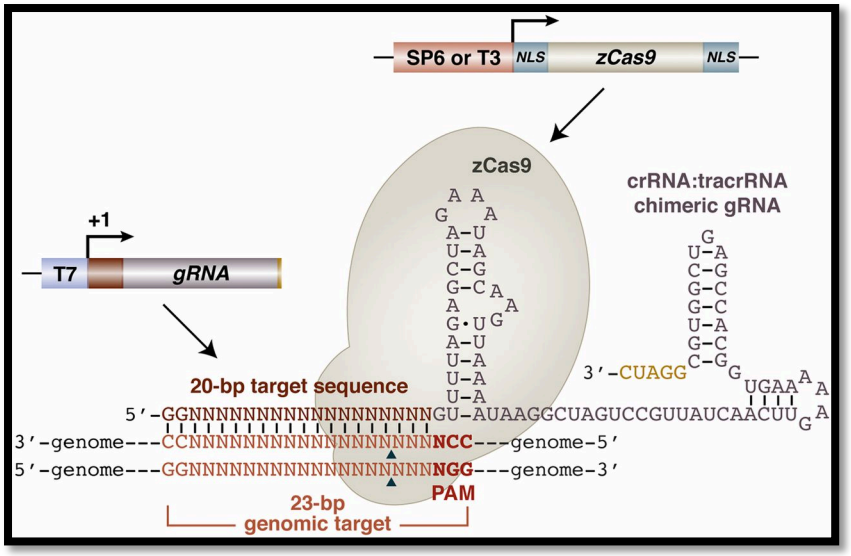

Figure 2. (taken from Figure 1, Jao et al., 2013) The CRISPR-Cas9 system is a reverse genetics approach to targeted mutagenesis. This system works by the guide RNA recruiting the Cas9 endonuclease to the target sequence where it will induce a double stranded break. Like ZFNs and TALENs, this double-stranded break can result in mutations by error-prone NHEJ.

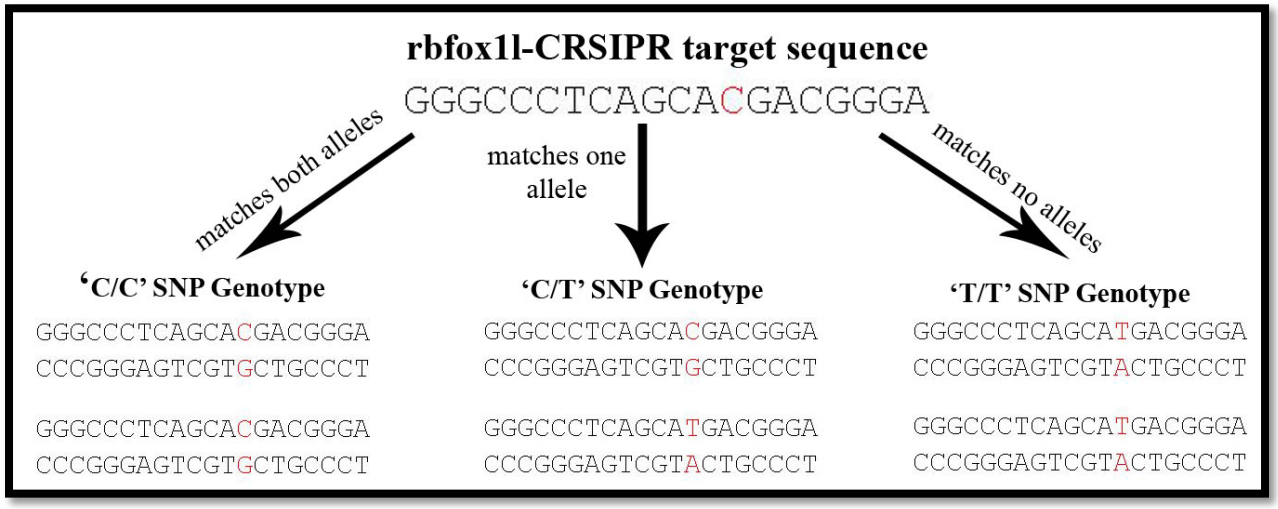

Figure 3. The rbfox1l guide RNA targeted a sequence that contained a SNP of a 'C' or 'T' that is present in wild-type fish. The guide RNA matches both alleles of the 'C/C' fish, one allele of the 'C/T' fish, and no alleles of the 'T/T' fish.

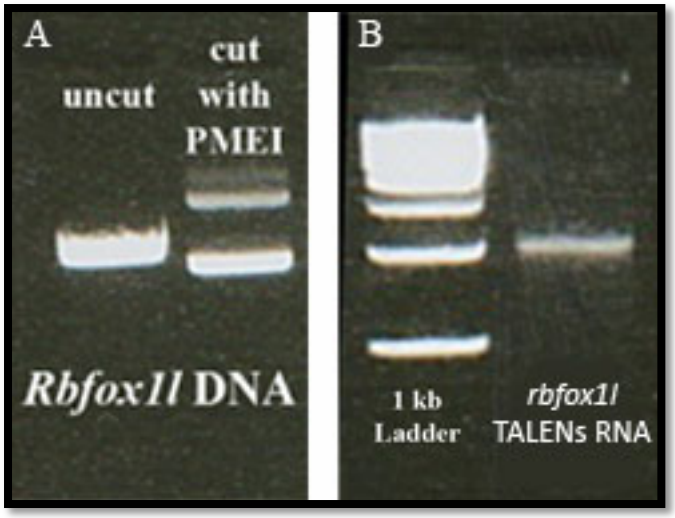

Figure 4. TALENs were used for targeted mutagenesis of the rbfox1l gene. (A) The TALENs system relies on inducing double-stranded breaks in the region complementary to the targeting sequences. The non-homologous end joining tends to be error prone and can thus lead to mutations. (B) The rbfox1l DNA was cut with PMEI and yielded the expected bands. (C) The rbfox1l RNA was run on a diagnostic gel and was observed to run in a single, bright band.

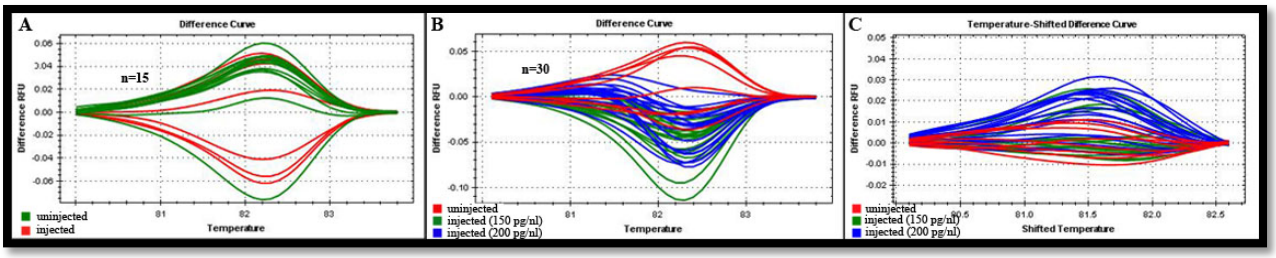

Figure 5. Embryos injected with *rbfox1l* TALENs do not show great deflection from uninjected wild type embryos when analyzed via HRMA. (A) Embryos injected with 5 nl of rbfox1l TALENs (150 pg/nl) fell into the same clusters as the uninjected wild-type embryos. No sample showed significant deflection. (B) Additional embryos injected with *rbfox1l* TALENs (150 pg/nl and 200 pg/nl) still did not show great deviation from the uninjected wild-type embryos. Some deviation as great as 0.1 was observed but deflections did not exhibit a dosing curve as embryos injected with 200 pg/nl did not show deflection greater than 0.07. (C) When temperature shifted the difference curves between the injected and the uninjected embryos were seen to collapse together, further suggesting a lack of mutagenesis.

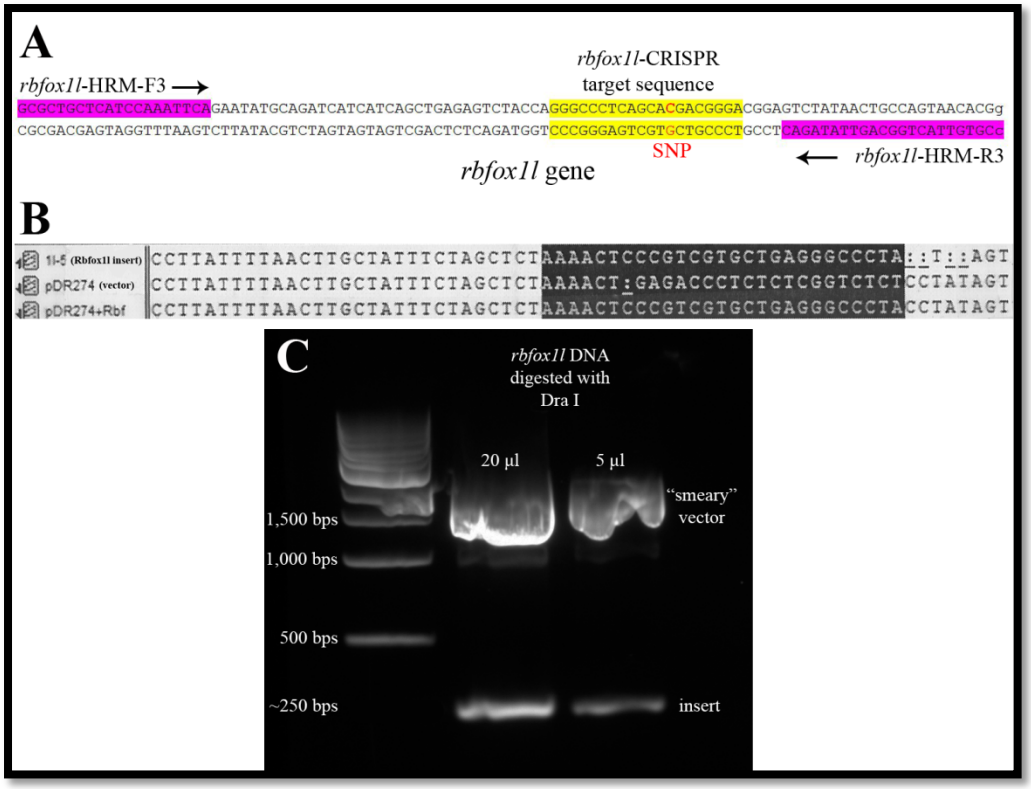

Figure 6. The *rbfox1l* gene was targeted for mutagenesis by the CRISPR-Cas9 system. (A) A 20 bp segment (shown in yellow) in exon 2 of *rbfox1l* was targeted by the CRISPR-Cas9 system. Primers (shown in pink) were designed to amplify this region for HRM analysis. The base pair in red represents a SNP in the zebrafish genome. (B) Sequencing of the plasmids confirmed that the rbfox1l insert had been successfully ligated into the pDR274 vector. (C) Gel analysis of a DraI digest of the rbfox1l guide DNA showed a tight band around 250 base pairs and a "smeary" band around 1,500 base pairs as expected to represent the rbfox1l insert and the larger vector, respectively.

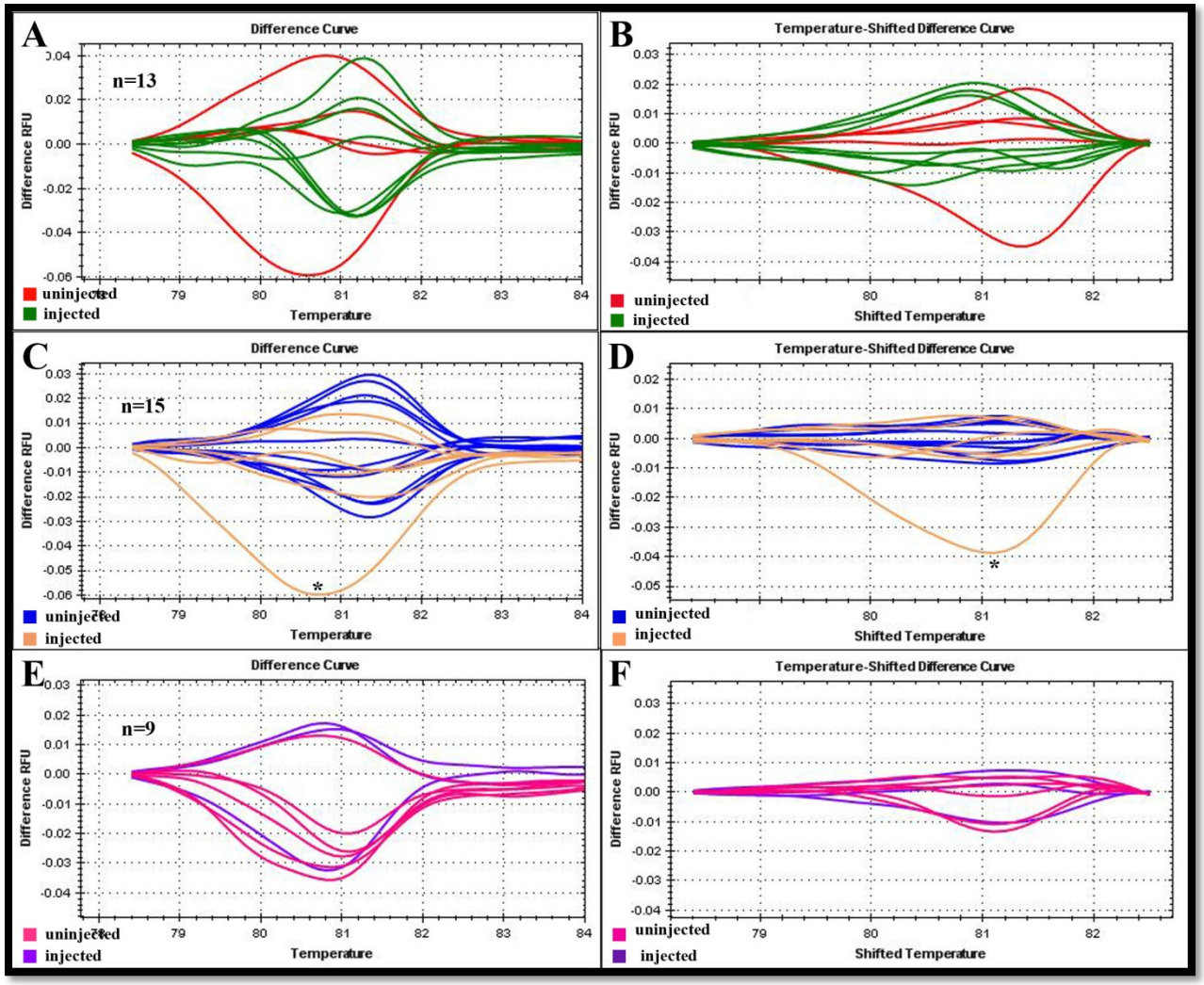

Figure 7. Embryos injected with rbfox1l-CRISPR (33 ng/µ/) and Cas9 (6 ng/µl) show very little deflection from wild-type. A) The injected embryos (green) show no deflection from uninjected (red). (C) One injected embryo (denoted by '*') shows mild deflection from uninjected embryos (blue). The remaining injected embryos (peach) show no deflection. (E) No injected embryos (purple) deflect from the uninjected controls (pink). (B, D, F) Temperature shifted view of HRM analysis further confirmed very little deflection of injected embryos from wild type. The embryo that showed mild deflection in the second SNP genotype continues to show some mild deflection when temperature shifted.

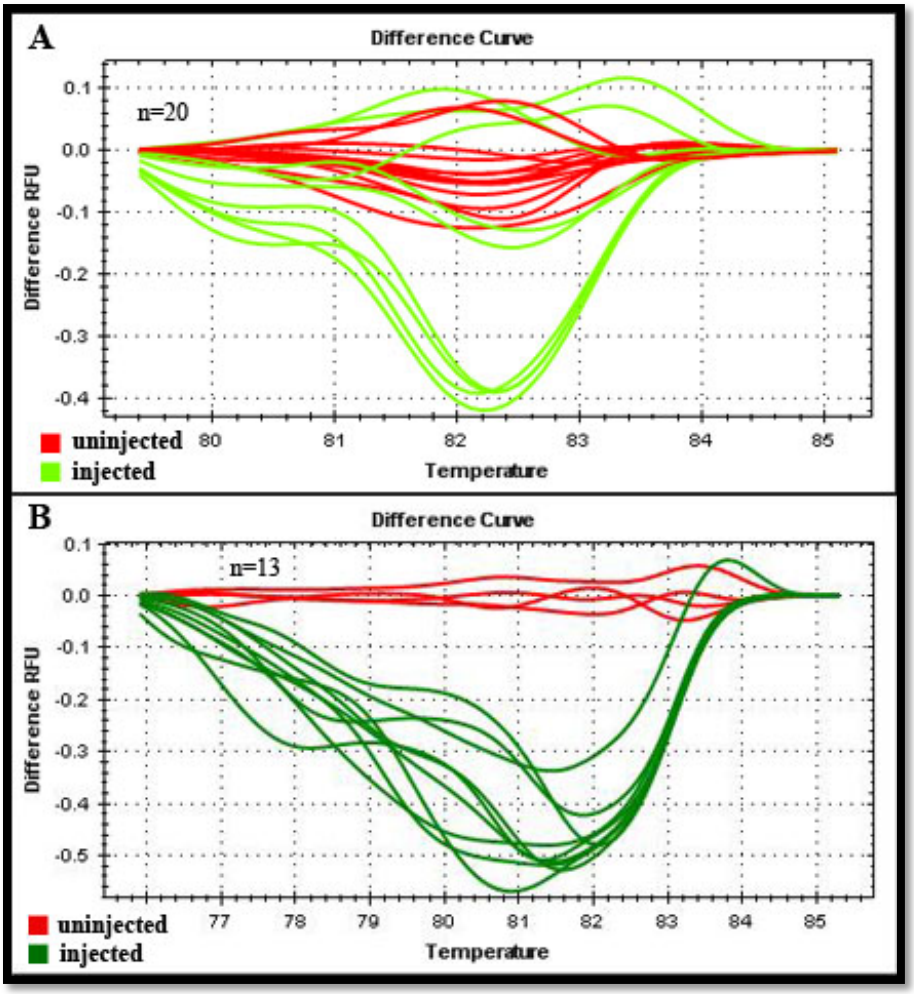

Figure 8. Since a *pnrc2*-CRISPR was known to produce strong deflections, it was used to test the Cas9 mRNA. (A) A previous injection of *pnrc2*-CRISPR with known Cas9 mRNA showed strong deflection in HRMA. (B) The test injection of *pnrc2*-CRISPR and unknown Cas9 mRNA produced strong deflection in HRMA, indicating that this Cas9 stock was functioning properly.

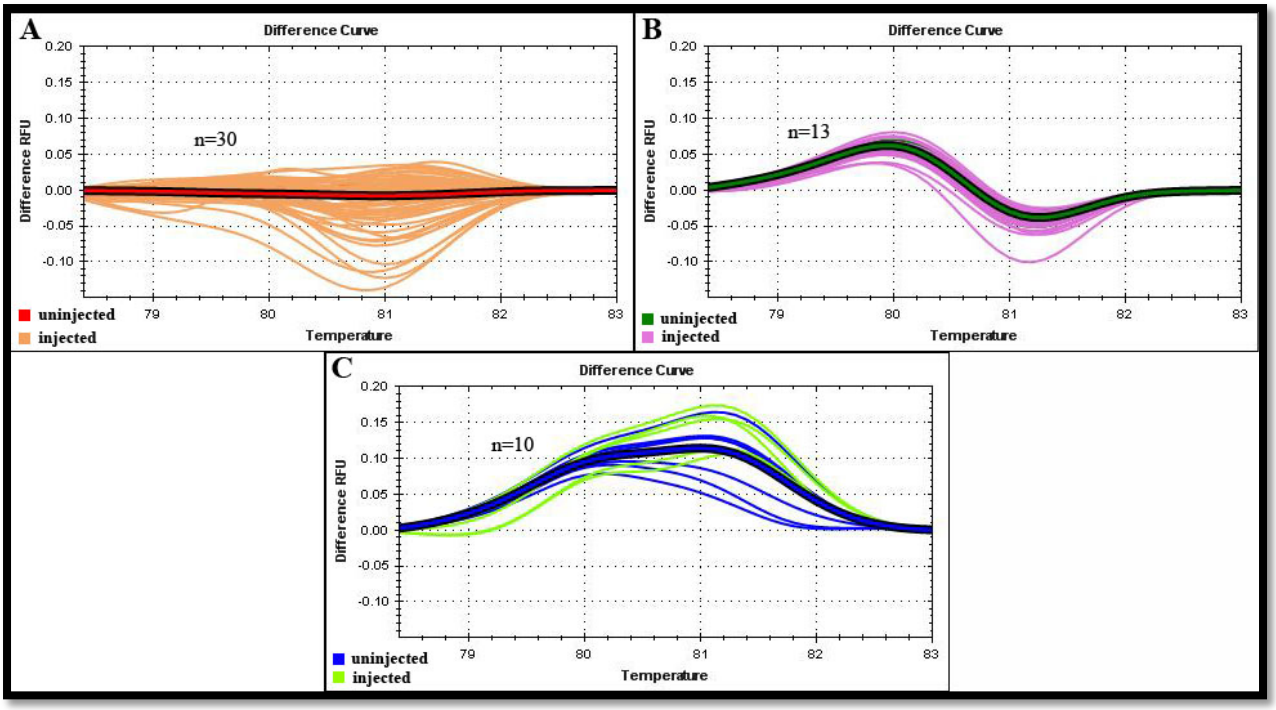

Figure 9. Injections of *rbfox1l*-CRISPR suggest that the CRISPR-Cas9 system may be more effective at targeting one SNP genotype than the others. All three HRMA difference curves are shown for each respective genotype. (A) The embryos injected of this SNP genotype (designated genotype 9A) appear to show consistent mild deflection from their uninjected wild type counterparts and thus may be more susceptible to mutagenesis than those injected embryos of the other two SNP genotypes. (B) Embryos injected from SNP genotype 9B do not appear to show great deflection from the uninjected samples. The greatest deflection is around 0.05 RFU, which is considered very weak deflection and not indicative of mutagenesis. (C) Although great variability is observed in the embryos of SNP genotype 9C, there is no consistent deflection of the injected embryos from the uninjected wild-type embryos.

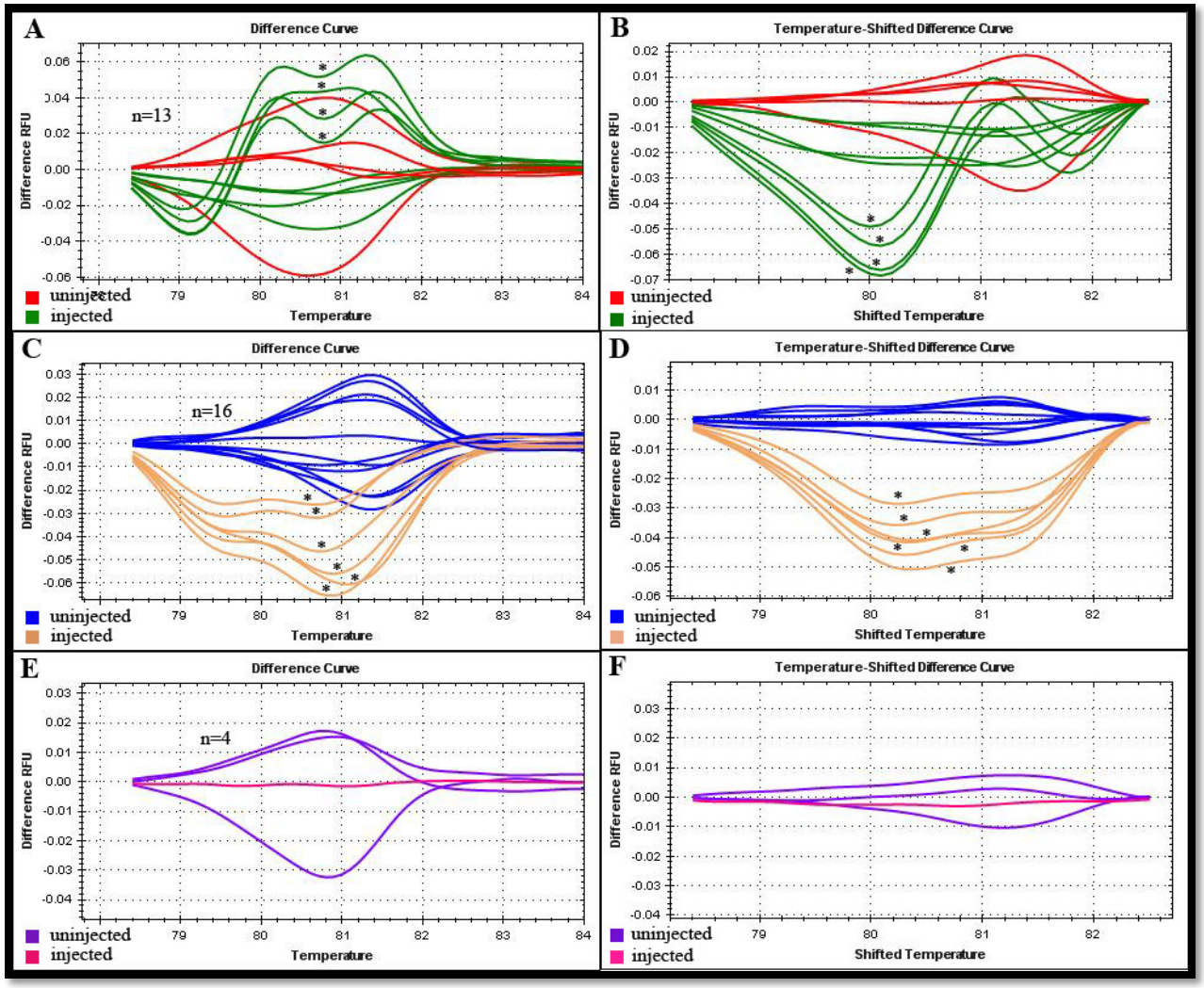

Figure 10. Embryos injected with rbfox1l-CRISPR (70 ng/µl) and Cas9 RNA (66 ng/µl) appear to show evidence of mutagenesis in two of the SNP genotypes. (A-B) Mutagenesis may be evident in four (denoted by '*') out of the eight embryos analyzed. Although the curves of these embryos do not deflect strongly the pattern of the curves deviates very sharply from the wild-type. This deviation is maintained when the samples are temperature shifted, further supporting mutagenesis. (C-D) Mutagenesis may be found in all six of the embryos (denoted by '*') screened as the HRMA curves have patterns that are very different from wild-type. This difference once again is maintained in the temperature shifted difference curves. (E-F) No deflection from wild-type is observed in this genotype. In fact, wild-type appear to differ more from each other than the injected sample.

Figure 11. The *rbfox2* gene was targeted for mutagenesis using the CRISPR-Cas9 system. The target sequence is shown is yellow. The primers used to amplify this region for HRMA are shown in pink.

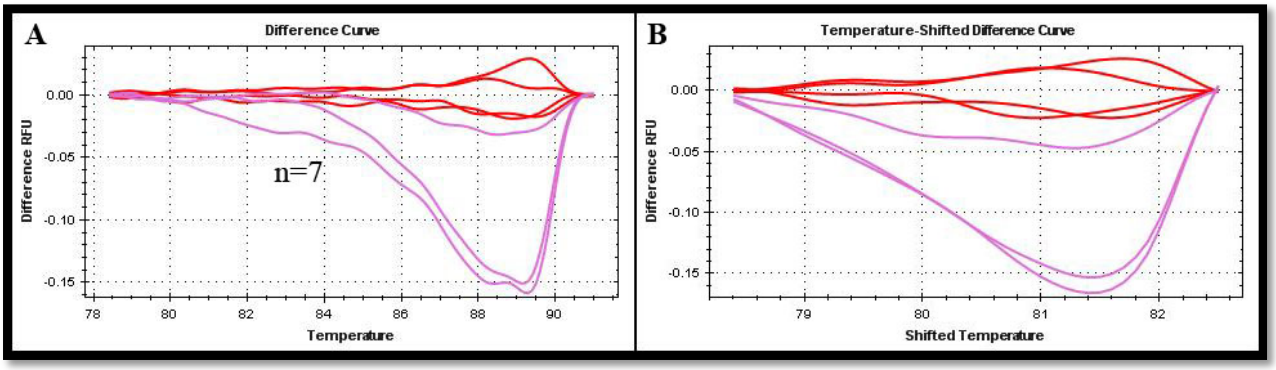

Figure 12. Embryos injected (pink) with *rbfox2*-CRISPR deflect significantly enough from wild-type (red) to indicate mutagenesis. (A) Two of the ten analyzed samples deflect from wild-type by at least 0.15 RFU, a value high enough to indicate that the CRISPR induced a change at the *rbfox2* locus. (B) The temperature shifted analysis of these embryos further supported that these samples had a mutation. Note that only three of the injected samples amplified well enough to analyze.

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